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Serotyping and Virulence Genes Detection in *Escherichia coli* Isolated from Fertile and Infertile Eggs, Dead-in-Shell Embryos, and Chickens with Yolk Sac Infection

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SUMMARY. *Escherichia coli* is a common avian pathogen mainly associated with extraintestinal infections such as yolk sac infection (YSI). The aim of this study was to determine the serotypes and the presence of some virulence genes of *E. coli* strains isolated from different samples in a vertically integrated poultry operation in Mexico. Two hundred sixty-seven *E. coli* isolates from different samples were serotyped using rabbit serum against the 175 somatic (O) and 56 flagellar (H) antigens of the typing schema. Virulence genes were determined by colony blot hybridization, using DNA probes for *st, eae, agg1, agg2, bfp, lt, cdt, slt,* and *ipaH* diarrhea-associated virulence factors. The serogroup of 85% of the strains was determined; O19 (12%), O84 (9%), O8 (6%), and O78 (5%) were the most common. Using the complete antigenic formula (O and H), O19:NM ($n = 31$) was the serotype most frequently isolated from dead-in-shell embryos and in broilers that had died on the fourth, fifth, sixth, and seventh days after hatch. One hundred ten strains (41.2%) hybridized with one or more of the used probes. Of these, *ipaH* (72%), *eae* (30%), and *cdt* (27%) were the most common. Considering the origin of the respective isolates, 40% of the broiler farm strains were positive for at least one probe. Results show that some avian *E. coli* strains isolated in Mexico are included in avian pathogenic *E. coli* serotypes not previously reported, suggesting that they could be specific for this geographic area. The wide distribution of the *ipaH* gene among nonmotile strains suggests that this invasiveness trait could be important in YSI pathogenesis. On the other hand, some other genes could contribute to *E. coli* virulence during YSI.

RESUMEN. Serotipificación y detección de genes de virulencia de aislamientos de *Escherichia coli* obtenidos a partir de huevos fértiles e infértiles, embriones muertos y pollos con infección del saco vitelino.

Escherichia coli es un patógeno aviar común asociado principalmente con infecciones extraintestinales tales como la infección del saco vitelino. Se determinó el serotipo y la presencia de algunos genes de virulencia de cepas de *E. coli* aisladas a partir de diferentes muestras obtenidas en una empresa avícola integrada de México. Se serotipificaron 267 aislamientos de *E. coli* obtenidos a partir de las diferentes muestras empleando suero de conejo contra los antígenos somático (O) 175 y flagelar (H) 56 del esquema de tipificación. Se determinaron los genes de virulencia en las colonias de *E. coli* mediante hibridación de punto con sondas de ADN dirigidas contra los factores de virulencia asociados con diarrea *st, eae, agg1, agg2, bfp, it, cdt, slt* e *ipaH*. Se determinó el serogrupo del 85% de las muestras, siendo los serogrupos más comunes el O19 (12%), O84 (9%), O8 (6%) y el O78 (5%). Mediante el uso de la fórmula antigénica completa (O y H), se determinó que el serotipo aislado con mayor frecuencia a partir de embriones muertos y de pollitos de engorde muertos a los 4, 5, 6 y 7 días posteriores al nacimiento fue el O19 (31 aislamientos). Un total de 110 cepas (41.2%) hibridizaron con una o mas de las sondas empleadas. De estas, *ipaH* (72%), *eae* (30%) y *cdt* (27%) fueron las mas comunes. Considerando el origen de los aislamientos respectivos, el 40% de las cepas aisladas a partir de los pollos de engorde fueron positivas por lo menos a una sonda. Los resultados muestran que algunas cepas aviares de *E. coli* aisladas en México se encuentran incluidas en los serotipos patógenos aviares de *E. coli* no reportados con anterioridad, sugiriendo que estos aislamientos podrían ser específicos de esta zona geográfica. La amplia distribución del gen *ipaH* entre las cepas no móviles sugiere que esta característica de invasividad podría ser importante en la patogénesis de la infección del saco vitelino. Sin embargo, otros genes podrían contribuir a la virulencia de *E. coli* durante la infección del saco vitelino.

Key words: *Escherichia coli*, yolk sac infection, serotyping, DNA hybridization, virulence genes

Abbreviations: A/E = attaching and effacing; APEC = avian pathogenic *Escherichia coli*; BFP = bundle-forming pilus; CDT = cytolethal distending toxin; EAEC = enteroaggregative *Escherichia coli*; EAF = entero adherent factor; EHEC = enterohemorrhagic *Escherichia coli*; EIEC = enteroinvasive *Escherichia coli*; EPEC = enteropathogenic *Escherichia coli*; ETEC = enterotoxigenic *Escherichia coli*; H = flagellar antigen; H? = H nontypeable; HUS = hemolytic uremic syndrome; IpaH = invasion plasmid antigen H; LT = heat-labile toxin; O = somatic antigen; O? = O nontypeable; OR = O rough; SLT = *Shiga*-like toxin; ST = heat-stable toxin; YSI = yolk sac infection

Bacterial infections are of worldwide importance in commercially produced poultry, and their costs exceed \$100 million annually (4,17,44). *Escherichia coli* that cause disease in chickens are collectively known as avian pathogenic *Escherichia coli* (APEC) (29) and have been mainly associated with extra-intestinal infections such as air sacculitis, pericarditis, peritonitis, salpingitis, synovitis, osteomyelitis, cellulitis, and yolk sac infection (YSI) (13,21, 29,44).

Before the use of *E. coli* serologic typing, the isolation of these bacteria from avian products was considered as external contamination. However, with the use of typing and some other procedures, the etiologic significance of *E. coli* in avian diseases has been assessed (19). Studies (2,21,27,41) have shown that O1, O2, O78, and O nontypeable (O?) are the serogroups most frequently associated with poultry.

Characteristics such as specific serotypes, colicin V production, fimbriae F1 type expression, embryo lethality, *iss* genetic element, temperature-sensitive hemagglutinin Tsh, host complement resistance, polysaccharide capsule, aerobactin iron-sequestering systems, and toxin or cytotoxin production are common features of APEC strains (12,13,17,18,28).

Escherichia coli is the most predominant facultative anaerobe of the colonic flora. However, several clones have evolved the ability to cause a broad spectrum of human diseases (36). Diarrheagenic *E. coli* strains include five major groups: enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enterohemorrhagic *E. coli* (EHEC), and enteroinvasive *E. coli* (EIEC) (31,36). The versatility of *E. coli* is due to the fact that different strains have horizontally acquired different virulence genes (45).

EPEC strains have been associated with acute and persistent diarrhea in the developing world among infants younger than 2 years. Although EPEC-like organisms associated with disease have been isolated from animals such as rabbits, pigs, and dogs, the serotypes found in these animals are usually not human serotypes (36). The hallmark of the infection due to EPEC is the attaching and effacing (A/E) histopathologic condition, which is characterized by effacement of microvilli and intimate adherence between the bacterium and the epithelial cell membrane. Intimin, an outer membrane protein, is an adhesin encoded by the *eae* gene (for *E. coli* A/E), and it is present in all EPEC and EHEC strains that produce the A/E lesion. The *eae* gene is essential for the virulence of EPEC strains, although additional virulence factors such as the bundle-forming pilus (BFP) are important in the virulence of these strains. The BFP is encoded by the structural gene *bfpA* (encoded on the entero adherent factor [EAF] plasmid) and is responsible for the localized adherence to HEp-2 cells (36,45).

First recognized as a cause of diarrheal disease in piglets, ETEC strains are now considered as a major cause of disease in children in developing countries and in adults traveling to ETEC-endemic (traveler's diarrhea) areas (45). The ETEC group is defined as *E. coli* strains that elaborate at least one of a defined group of enterotoxins, including heat-stable toxin (ST) and heat-labile toxin (LT); in both, the encoding genes are on plasmids (36,45).

The EAEC strains have been associated with persistent and bloody diarrhea in developing countries and some developed countries (45). The characteristic aggregative adherence pattern of EAEC is associated with the presence of large plasmids. In addition to their role in the pathogenesis of diarrheal disease, DNA probes that hybridize against fragments from those plasmids have been used as a rapid and sensitive genotypic screening technique for EAEC detection and diagnosis of EAEC infections (45,47).

EHEC were recognized as an emerging pathogen in 1982 in the United States (31). At that time, the rarely isolated serotype O157:H7 was identified in two outbreaks of hemorrhagic colitis associated with the ingestion of undercooked hamburgers at a fast-food restaurant chain. EHEC strains cause A/E lesions on epithelial cells and possess a 60-MDa plasmid (31,36). However, the major virulence factors and defining characteristics of EHEC are the *Shiga*-like toxins 1 and 2 (SLT1 and SLT2) associated with the hemolytic uremic syndrome (HUS) in developed countries (36). Although SLT-producing *E. coli* can be found in the fecal flora of a variety of animals, cattle are considered the most important reservoir in terms of human infection because most cases have been caused by ingestion of foods of bovine origin (36).

EIEC strains are closely related to *Shigella* species in biochemical, genetic, and pathogenic properties. Organisms have been shown to invade the colonic epithelium, a phenotype mediated by plasmid and chromosomal loci. The genes for invasiveness are carried on a 120-MDa plasmid in *Shigella* serotypes and in EIEC strains (36). ipaH is an invasion plasmid antigen that, unlike others such as virA (30), is present in multiple copies (in the invasion plasmid and the chromosome) (50).

Cytolethal distending toxin (CDT) was first described in certain *E. coli* strains (56); however, it is present in a large spectrum of gram-negative bacterial species such as *Campylobacter* species, *Shigella dysenteriae*, and *Haemophilus ducreyi* (1,11,36,42). This toxin induces characteristic elongation of eukaryotic cells followed by progressive cellular distension and death. Because this toxin is produced by several bacterial pathogens, it can be hypothesized that CDT is a virulence factor that enhances the ability of a specific strain to cause damage (36). CDT activity is encoded by three closely linked genes termed *cdtA, cdtB,* and *cdtC*; these genes are adjacent on a large virulence plasmid (42).

Different virulence factors have been detected within APEC strains, including several virulence markers of human diarrheagenic groups. Despite the enormous amount of knowledge regarding avian *E. coli* in some countries, the incidence of serotypes and the distribution of virulence genes among APEC strains in Mexico are unknown. This is one of the first attempts to determine the predominant serogroups of APEC strains in Mexico. Results from a previous investigation on the role of *E. coli* in YSI showed that this bacterium was the most frequently isolated from different samples (43). The aim of this study was to determine the most common serotypes and identifying virulence genes associated with diarrheagenic groups among *E. coli* strains isolated from cases of YSI in a vertically integrated poultry operation.

MATERIALS AND METHODS

Bacterial identification and selection. To identify the origin of YSI, a batch of fertile eggs was followed from the breeder farm through the hatchery and to the broiler farm in a vertically integrated poultry operation located at Guanajuato State (43). Briefly, samples from sawdust of the nest (n = 60), non-disinfected fertile eggs at the breeder farm (n = 60), infertile eggs (n = 60), dead-in-shell embryos at the hatchery (n = 60), and first-week mortality associated with YSI (n = 216) were analyzed (5,14,43). From these, 267 *E. coli* isolates were identified (Table 1). In the present article, those *E. coli* strains were analyzed to determine their serologic identity and the presence of virulence genes associated with YSI.

Serotyping. Rabbit sera prepared against 175 somatic (O) and 56 flagellar (H) *E. coli* antigens (SERUNAM, Mexico City, Mexico), were used for serotyping as previously described by Orskov and Orskov (37). Forty-seven specific rabbit sera prepared against the different *Shigella* serogroups and three more prepared against Mexican *E. coli* strains that do not react with the recognized antiserum scheme were used.

DNA hybridization. The 267 *E. coli* strains were analyzed by colony dot hybridization using nine DNA probes designed for *E. coli* diarrheal-associated virulence factors (Table 2). All the probes were synthetic oligonucleotides (custom primers; GIBCO BRL, Gaithersburg, MD). The probes were labeled at the 5′ end by the enzymatically catalyzed transfer of ^{32}P from [γ-^{32}P] ATP (ICN Pharmaceuticals, Irvine, CA) with T4 polynucleotide kinase (Bethesda Research Laboratories, Gaithersburg, MD), according to the procedure described by Sambrook *et al.* (46). The strains were cultured overnight in brain heart infusion at 37 C and spot-inoculated onto MacConkey agar plates (CM7B; Oxoid Inc., New York). Positive and negative controls were inoculated in each plate

Table 1. Isolation source of *E. coli* strains and positive DNA hybridization against different virulence probes.

Origin	No. of strains	Probes (No. of positive strains)						
		ipaH	*eae*	*cdt*	*ipaH-eae*	*ipaH-cdt*	*eae-cdt*	*ipaH-eae-cdt*
Breeder farm								
Fertile eggs	4	2						
Hatchery								
Infertile eggs	28	8	1	1	4	1		
Dead in shell	47	3		5				
Broiler farm								
Liver[A]	102	21	7	5	3	2	1	6
Yolk sac[A]	86	17	7	2	7	3	1	3
Total	267	51	15	13	14	6	2	9

[A]Organs were obtained from first-week mortality at broiler farm.

according to the corresponding tested probe (Table 3). After overnight incubation at 37 C, the colonies were replicated by colony lift onto circular membranes (No. 541, Whatman, Clifton, NJ) and then lysed using the procedure described by Hill *et al.* (25).

A 50-ml hybridization mixture was prepared in a plastic tube just before use (25). One milliliter of salmon sperm was boiled for 10 min and added to the hybridization mixture. Five to 10 ml were dispensed into a plastic Petri dish containing the paper filters with the lysed bacterial colonies. The volume of the DNA-labeled probe solution was adjusted to contain 1×10^6 counts per minute, and this was added to the hybridization solution containing the filters, mixed briefly, and incubated overnight at 41 C. The filters were removed from the hybridization mixture and rinsed for 5 to 10 sec in plastic Petri dishes containing 10 ml of $6 \times$ SSC at 54 C and washed to eliminate the free ^{32}P-labeled DNA probe, which was not specifically bound to the DNA of the lysed colonies. Filters were drained and covered again with $6 \times$ SSC and incubated for 1 more hour at 50 C, at which time the filters were again drained, covered with $6 \times$ SSC, and incubated for 1 more hour. Finally, the filters were rinsed for 5 to 10 sec in $2 \times$ SSC and dried at room temperature. Results were visualized by autoradiography on film (XAR-2 in 8 $\times$ 10-inch size; Eastman Kodak, Rochester, NY) with intensifying screen cassettes and exposing during 48 hr at −70 C and developing according to the manufacturer's instructions.

RESULTS

Serotyping. Forty-two different serogroups were identified among the 267 *E. coli* isolates

Table 2. Oligodeoxyribonucleotide probes used to identify *E. coli* virulence genes.

Virulence factor	Probe sequences	Source
st1[A]	5 -GCTGTGAATTGTGTTGTAATCC-3	Murray *et al.* (35)
*eae*1[B]	5 -ACGTTGCAGCATGGGTAACTC-3	Blanco *et al.* (6)
agg1[C]	5 -CTGGCGAAAGACTGTATCAT-3	Schmidt *et al.* (47)
agg2[C]	5 -CAATGTATAGAAATCCGCTGTT-3	Schmidt *et al.* (47)
bfp[D]	5 -GCTACGGTGTTAATATCTCTGGCG-3	Nataro and Kaper (36)
lt-A[E]	5 -GCGAGAGGAACACAAACCGG-3	Schultsz *et al.* (48)
cdt[F]	5 -CACGTCTGCAAGGCACTAC-3	Scott and Kaper (49)
ipaH[G]	5 -CTGGAGGACATTGCCCGGG-3	Venkatesan *et al.* (57)
slt[H]	5 - GATGATCTCAGTGGGCGTTC -3	Gunzer *et al.* (22)

[A]Heat-stable enterotoxin gene.
[B]*eae* Intimin gene.
[C]60–65-kDa plasmid.
[D]Bundle-forming pilus gene.
[E]Heat-labile enterotoxin gene.
[F]Cytolethal distending toxin gene.
[G]Invasion plasmid antigen H gene.
[H]*Shiga*-like toxin gene.

studied. The serogroups O19 (12%), O84 (9%), O8 (6%), and O78 (5%) were the most common (Table 4). The serogroup was not determined in 15% of the isolates; of these, 11% were rough (OR) and 4% were O?.

The association between source of isolation and serogroup shows that O6, O9, and O152 *E. coli* strains were common in the breeder and broiler farms; O125 was identified only at the breeder farm and hatchery, whereas O3, O24, and O146 were isolated only at the hatchery (Table 5). In infertile eggs, the identified serogroups were O69, O70, O153, and O167, whereas O41 and O85 were found in dead-in-shell embryos. Twenty different serogroups were determined in strains recovered from the first-week broiler mortality (Table 5). The most frequently identified serogroups associated with YSI in broilers were OR, O19, O78, and O84, strains; of these, the latter two were isolated only at the broiler farm, whereas serogroups OR and O19 were isolated from broiler farm and hatchery samples.

During the identification of the flagellar antigens, it was observed that 30% of the strains were nonmotile. Of the motile strains, the most common were identified as possessing H:8 (12%), H nontypeable (H?) (9%), H:10 (8%), H:31 (6%), and H:11 (6%). The characterization of the strains using the complete antigenic formula showed that O19:NM ($n = 31$) was the most common serotype isolated from dead-in-shell embryos and from broilers that died on the fourth, fifth, sixth, and seventh days after hatch. Another common serotype was OR:NM ($n = 16$), isolated from infertile eggs and during the third mortality day of broilers.

DNA hybridization. One hundred ten strains (41.2%) hybridized with one or more of the follow probes: *ipaH, cdt,* and *eae* (Table 6). Of these, 80 strains were *ipaH* positive (72%), 40 were *eae* positive (30%), and 30 were *cdt* positive (27%). Some of these strains hybridized with more than one gene, showing different profiles; the most frequently observed was *ipaH-eae* (13%). Positive strains to *st1, agg1, agg2, bfp, lt-A*, and *slt* probes were not found.

The correlation between serogroup and gene hybridization shows that O78 ($n = 6$) and O84 ($n = 6$) serogroups and OR strains ($n = 19$) were most frequently associated with the *ipaH* gene (Table 4); similarly, those serotypes showed a high correlation with the *eae* gene (4, 8, and 11 strains, respectively). On the other hand, the *cdt* gene was identified mainly on OR ($n = 13$), O19 ($n = 4$), and O2 ($n = 3$).

The correlation between origin of the strains and DNA hybridization shows that four strains were isolated from the breeder farm, but only two of them were positive for the *ipaH* probe (Table 1). Regarding samples from the hatchery, many of the strains isolated from infertile eggs and from dead-in-shell embryos were also positive in the hybridization test. For the samples from the broiler farm, more than 40% of the strains were positive for at least one probe. The gene association *ipaH-eae-cdt* was identified in the strains isolated from the liver and yolk sac; however, the same gene combination was not observed in previous stages of production. Considering the association between the occurrence of the genes and mortality, the fifth day was when most of the strains carrying the *ipaH* gene were isolated (Table 6), although the *ipaH*-positive strains were isolated from the third until the seventh days of mortality. The *ipaH* gene was not observed before the third day of mortality (Table 6). The *cdt* gene was identified in the strains isolated during the last incubation stage (dead-in-shell embryos) and in the first 4 days in the broiler house, but not later.

Table 3. Positive and negative controls of *E. coli* strains used in the DNA hybridization assay.

Strain	Serotype	Group	Genes
E3787	O26:H11	EHEC	*slt1*
E32511	O157:H−	EHEC	*eae1*
E2347	O127:H6	EPEC	*eae1, bfp, cdt*
E60725	O92:H33	EAEC	*agg1*
E66438	O75:H−	EAEC	*agg2*
E7476	O166:H27	ETEC	*st1*
E5798	O7:H18	ETEC	*ltA*
E35990	O143:H−	EIEC	*ipaH*
14R519	K12	None	Negative control

DISCUSSION

YSI is considered the major cause of first-week mortality in broilers (3,10,20). YSI occurs when fertile eggs are contaminated, mainly by *E. coli* (3,10,23,33). One of the more common difficulties in diagnosing and controlling avian colibacillosis is that specific virulence markers have not been found in APEC strains (15,26).

Although more than 50,000 different *E. coli* serotypes exist, only a few are associated with disease (38). For this reason, the use of serotyping could continue to help to determine the epidemiologic importance of APEC strains in different avian diseases.

Nontypeable strains of APEC are common; studies (2,7,9,19,24,27,41) have shown that be-

Table 4. Serotypes and virulence genes of *E. coli* strains isolated from different samples.[A]

Serotype	Serogroup	Total No. of analyzed strains	*ipaH*	*eae*	*cdt*	Serotype	Serogroup	Total No. of analyzed strains	*ipaH*	*eae*	*cdt*
O2:H1	6 (2.25)	1	+	−	−	O85:NM	2 (0.75)	2	−	−	−
O2:H8		1	+	+	−	O91:H7	4 (1.50)	2	−	−	−
O2:NM		2	+	−	+	O91:H7		1	−	−	+
O2:NM		1	+	+	+	O91:H7		1	+	−	−
O2:NM		1	+	−	−	O103:H11	10 (3.75)	6	−	−	−
O3:H1	2 (0.75)	1	+	−	−	O103:H11		1	+	−	−
O3:NM		1	+	−	−	O103:NM		3	−	−	−
O4:H11	1 (0.37)	1	−	−	−	O108:H19	3 (1.12)	1	−	−	−
O6:H16	2 (0.75)	1	+	−	−	O108:H?		1	−	−	−
O6:H?		1	+	−	−	O108:NM		1	−	−	−
O7:H18	2 (0.75)	2	−	−	−	O112:H19	6 (2.25)	1	−	+	−
O8:H9	17 (6.37)	1	−	−	−	O112:H19		1	−	−	−
O8:H19		4	−	−	−	O112:H51		2	−	−	−
O8:H49		3	−	−	−	O112:H51		2	+	−	−
O8:H?		3	−	−	−	O118:H31	7 (2.62)	4	+	−	−
O8:H?		1	+	−	−	O118:H31		3	−	−	−
O8:NM		1	−	−	+	O120:H10	11 (4.12)	8	−	−	−
O8:NM		4	−	−	−	O120:H25		1	+	−	−
O9:H11	6 (2.25)	3	−	−	−	O120:H25		1	−	−	−
O9:H11		1	+	−	−	O120:NM		1	−	−	+
O9:H11		1	−	+	−	O124:H10	1 (0.37)	1	+	−	−
O9:H25		1	−	−	−	O125:H8	3 (1.12)	2	+	−	−
O12:H31	3 (1.12)	2	−	−	−	O125:H8		1	−	−	−
O12:H31		1	−	+	−	O132:H42	1 (0.37)	1	−	−	−
O15:H9	6 (2.25)	1	−	−	−	O146:H19	5 (1.87)	3	−	−	−
O15:H10		3	−	−	−	O146:H19		1	+	−	−
O15:H44		1	−	−	−	O146:H28		1	−	−	−
O15:H45		1	−	−	−	O149:H23	2 (0.75)	1	−	−	+
O19:H8	33 (12.36)	1	−	−	−	O149:H23		1	−	−	−
O19:H8		1	+	−	−	O152:H6	4 (1.50)	2	−	+	+
O19:NM		24	−	−	−	O152:H6		1	−	−	−
O19:NM		4	−	−	+	O152:H11		1	−	−	−
O19:NM		2	+	−	−	O153:NM	1 (0.37)	1	−	−	−
O19:NM		1	−	+	−	O155:H10	10 (3.75)	7	−	−	−
O21:NM	2 (0.75)	1	−	−	−	O155:H10		2	+	−	−
O21:NM		1	+	+	−	O155:H31		1	+	−	−
O22:H4	5 (1.87)	3	+	−	−	O167:H4	3 (1.12)	1	−	−	−
O22:H4		2	−	−	−	O167:H4		1	+	−	−
O23:H16	1 (0.37)	1	−	−	−	O167:H4		1	+	+	−
O24:H?	3 (1.12)	1	−	−	−	O168:H?	6 (2.25)	2	−	−	−
O24:H?		1	+	+	−	O168:H?		1	+	−	−
O24:NM		1	−	−	−	O168:NM		1	+	−	−
O41:H45	3 (1.12)	3	−	−	−	O168:NM		1	+	+	−
O44:NM	5 (1.87)	3	−	−	−	O168:NM		1	−	−	+
O44:NM		2	−	−	+	OR:H1	29 (10.86)	2	−	−	−
O53:H25	3 (1.12)	2	−	−	−	OR:H2		1	+	−	−
O53:NM		1	+	+	+	OR:H4		1	+	+	−
O69:H38	2 (0.75)	1	+	+	−	OR:H5		1	+	−	−
O69:H38		1	−	−	−	OR:H8		2	−	+	−
O70:H?	1 (0.37)	1	−	−	−	OR:H8		1	+	−	−
O73:H55	3 (1.12)	1	−	−	−	OR:H9		1	+	+	−
O73:NM		2	−	−	−	OR:H11		1	−	−	−

Table 4. Continued.

Serotype	Serogroup	Total No. of analyzed strains	*ipaH*	*eae*	*cdt*	Serotype	Serogroup	Total No. of analyzed strains	*ipaH*	*eae*	*cdt*
O77:H?	1 (0.37)	1	−	−	−	OR:H31		2	−	−	−
O78:H9	13 (4.87)	4	−	−	−	OR:H?		1	+	−	−
O78:H9		3	+	+	−	OR:NM		2	+	−	−
O78:H9		3	+	−	−	OR:NM		7	+	+	+
O78:H9		1	−	+	−	OR:NM		4	+	−	+
O78:H33		1	−	−	−	OR:NM		2	−	−	+
O78:H?		1	−	−	−	OR:NM		1	−	−	−
O81:H31	2 (0.75)	1	−	−	−	O?:H5	10 (3.75)	3	+	−	−
O81:H31		1	+	−	−	O?:H5		1	+	+	−
O83:H4	3 (1.12)	3	−	−	−	O?:H31		1	+	−	−
O84:H8	24 (8.99)	12	−	−	−	O?:H?		1	−	−	−
O84:H8		6	−	+	−	O?:NM		2	−	+	−
O84:H8		4	+	−	−	O?:NM		2	−	−	−
O84:H8		1	+	+	−						
O84:H31		1	+	+	−						

[A]Samples were nondisinfected fertile eggs, infertile eggs, dead-in-shell embryos, and mortality during the first week. Data are given as number (percentage) among 267 strains unless otherwise indicated.

tween 61% and 67% of the strains are nontypeable. In the present study, the serotype of 85% of the isolates was determined. A possible explanation for the high percentage of serogroups identified in our study could be the use of a complete scheme of recognized O antigens and the short period between the isolation and typing, as the identification was performed a few days after the isolation.

The existence of certain specific serotypes depends on conditions such as geographic area, country, and time of isolation (27,41). In a study (53) performed in Hokkaido, Japan, the researchers initially determined that O60, O53, and O1 were the most frequently isolated serogroups, whereas in a subsequent study, the predominant serogroups were O1, O2, O8, and O78 (53). Surveys in other countries have revealed that the most common serogroups isolated from sick chickens were O1, O2, and O78 (9,12,13,19,44,58,59). In Mexico, this is one of the first attempts to determine the predominant serogroups of APEC strains. Although O19 (12%) was the most common, O78 and O2 serogroups were also identified in our study. These results are consistent with the variability of serogroups and serotypes of APEC strains identified around the world (9,12,13,19,44,58,59).

The O1 serogroup has been related principally with septicemic processes (53). In our study, the O1 serogroup was not identified, possibly because none of the strains of this study were isolated from septicemic chickens.

Our data show that the serogroups isolated from infertile and dead-in-shell embryos at the hatchery were the same as those associated with mortality at the broiler farm. These observations agree with earlier reports that mention that, while the hatchery and the breeder farm could act as sources of bacterial contamination, the breeder farm is the main source for YSI (10,20,33).

EIEC causes a disease in humans that resembles bacillary dysentery. A common characteristic of these strains is that, like *Shigella* species, they have the ability to invade the epithelial cells of the colon (34). In fact, EIEC and *Shigella* strains are nonmotile bacteria that are biochemically and genetically related (36). Cloud *et al.* (9) reported that 41.6% of the *E. coli* strains isolated from the hatchery and from broilers were nonmotile. Similarly, a high percentage (71%) of *E. coli* isolates in the present study were nonmotile, suggesting that these could be related to EIEC strains.

ipaH is an invasion plasmid antigen that, unlike others such as virA (30), is present in multiple copies (in the invasion plasmid and the chromosome) (50); therefore, *ipaH* is often used as a diagnostic tool for EIEC detection. A high incidence (30%) of strains carrying the *ipaH* gene was observed in our study. This percentage is even

Table 5. Most common serogroups identified in *E. coli* strains isolated from different sources.

Serogroup	Eggs Fertile	Eggs Infertile	Dead-in-shell embryos	Broilers	Total
O2		1	1	4	6
O3		1	1		2
O6	1			1	2
O8			7	10	17
O9	1			5	6
O15				6	6
O19			20	13	33
O24		2	1		3
O41			3		3
O69		2			2
O78				13	13
O84				24	24
O85			2		2
O91				4	4
O103		2	1	7	10
O108			1	2	3
O118				7	7
O120			8	3	11
O125	1	2			3
O146		4	1		5
O149			1	1	2
O152	1			3	4
O155				10	10
O167		3			3
OR		4		25	29
O?		1		9	10
O22, O44				5	10
O70, O153		1			2
O112, O168		2		4	12
O7, O21, O81				2	6
O12, O53, O73, 83				3	12
O4,O23,O77,O124, O132				1	5

higher than in another study performed in human populations (40). The wide distribution of the *ipaH* gene among our strains, especially among nonmotile strains, suggests that invasiveness could play an important role in YSI pathogenesis.

The serotypes O28ac, O29, O42, O112 ac, O124, O136, O143, O144, O152, O159, O164, and O167 have traditionally been considered as EIEC strains (31,32,36,51,54). Studies (40,52) have demonstrated that the serotype identification alone is not sufficient to determine an *E. coli* strain as diarrheagenic. Although we recovered some serotypes traditionally belonging to EIEC, O112 ($n=6$), O124 ($n=1$), O152 ($n=4$), and O167 ($n=3$), only five of these strains were positive for the *ipaH* gene in the DNA hybridization. Conversely, numerous isolates belonging to serotypes not previously reported as EIEC were found to harbor the *ipaH* gene. However, as previously mentioned, the existence of specific serotypes depends on different conditions (27,41), and we acknowledge that these strains were isolated from a host different from those in which EIEC has been traditionally isolated (34).

Formal and Hornick (16) mention that a smooth lipopolysaccharide is required for penetration into and multiplication within epithelial cells. However, in our study, many rough strains were genotypically *ipaH* positive and positive in an *in vitro* invasiveness assay (data not shown).

The diarrheagenic EPEC group has been associated with infant diarrhea in the developing world (36). The presence of the *eae* gene, along with the absence of *sltx*, is sufficient to define EPEC, because

Table 6. Mortality and virulence genes in *E. coli* strains isolated from first-week mortality on broiler farm.[A]

Day	Mortality	*ipaH*	*cdt*	*eae*	*ipaH-cdt*	*ipaH-eae*	*cdt-eae*	*ipaH-cdt-eae*
1	4.17		3.53					
2	10.19							
3	21.30	1.18	3.53		5.88			10.59
4	11.11	1.18	1.18	2.35			2.35	
5	25.00	20.00		14.12		11.76		
6	17.13	10.59						
7	11.11	11.76						

[A]Data are given as percentages.

possession of this sequence correlates with the existence of the locus of enterocyte effacement pathogenicity island (36). Some other virulence markers are not conserved in all EPEC strains, such as EAF plasmid; for example, several strains in which *bfpA* is absent have been isolated (55). On the other hand, EPEC strains are included in certain recognized O:H serotypes (36). Nataro and Kaper (36) reported that, although EPEC-like organisms have been implicated in animal diseases, the serotypes found in those cases are usually not human serotypes. Similar results were seen in the present work, as only one isolate was identified as *eae* positive from 14 strains belonging to EPEC serogroups (O44 [$n = 5$], O112 [$n = 6$]. and O125 [$n = 3$]).

EPEC and EHEC strains harbor *eae* genes; however, only EHEC strains carry *slt*x genes (8,36). Forty isolates of this study were positive for the *eae* gene, but all were negative for the *sltx* gene. This observation suggests that our strains are typical EPEC strains. However, because two types of SLTs have been described (36), it is necessary to analyze both genes to demonstrate that our strains do not belong to the EHEC group. In a study by Kariuki *et al.* (29), it was mentioned that the APEC group mainly consists of EPEC and ETEC serovars. Our results are consistent with those observations, because 50 strains included within the reported ETEC serovars were identified. Of these O6 ($n = 2$), O8 ($n = 17$), O9 ($n = 6$), O15 ($n = 6$), O78 ($n = 13$), O85 ($n = 2$), O153 ($n = 1$), and O167 ($n = 3$) were the more common serogroups. ETEC strains elaborate at least one member of the two defined groups of enterotoxins: ST and LT. However, in our study, all the isolated strains were negative for *st* and *lt* genes in the DNA hybridization assay (36).

The frequency of *cdt*-positive strains was low (10%) in this study, compared with the number of strains harboring the *ipaH* and *ea*e genes. However, these data are consistent with another report (42). A correlation between serotype and CDT production has not been established. Pandrey *et al.* (39) recently reported that CDT production seemed to be a common feature of O86a and O127a *E. coli* strains. Our results show that *cdt-positive* strains were associated principally with O19 and OR strains (Table 4). Several investigations have been carried out to establish the pathogenic potential of this toxin, but statistically significant differences between healthy and diseased patients have not been found (1). In contrast to earlier results indicating that CDT-expressing *E. coli* strains belong to classic EPEC (39), we did not observe a preferential association of CDT with EPEC strains, because only 2 (both O44 strains) of our 11 strains belonging to the classic EPEC serogroups (O44, O112, and O12) carried the *cdt* gene (Table 4), although the production of CDT has been reported not to be limited to EPEC strains (56). Recently, Tóth *et al.* (56) determined that OR strains are able to produce CDT; this finding is similar to our observations, because one of the most important CDT-producing groups was OR. These authors identified a novel variant of CDT among intestinal and extraintestinal strains of human, avian, and porcine origin. At least four different types of CDT are known (56), and because a given strain is able to harbor only one of them; the prevalence of *cdt*-positive strains could be underestimated (56).

In the present work, the application of conventional and molecular approaches to the screening and characterization of *E. coli* strains was done. To our knowledge, this is the first systematic report that presents the prevalence of serotypes in the Mexican poultry industry. The serogroups identified among YSI-related strains have not been previously reported as pathogens in chickens, and some strains carried virulence genes that had been exclusively observed in humans. Moreover, our results suggest that the strains identified as causative agents of YSI in Mexico are endemic to this region. Although most of our strains carried the *ipaH* gene characteristic of invasive strains, suggesting that invasiveness could play a role in YSI, further characterization of these strains is needed to determine if the invasive phenotype is expressed and to clarify their specific participation in the pathogenesis of YSI.

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